

THE EFFECTS OF POLYPHOSPHATE ON CHEMOTAXIS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Effects of Polyphosphate on Chemotaxis

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Wound treatment in the United States costs \$25 billion annually (Sen, Gordillo et al. 2009) and one in a hundred people are hospitalized each year for a wound that requires professional medical attention (Drew, Posnett et al. 2007, Srinivasaiah, Dugdall et al. 2007, Hurd and Posnett 2009, Vowden and Vowden 2009, Vowden and Vowden 2009). It is not well understood why immune cells are brought together to the site of a wound. Determining how immune cells are signaled to the site of a wound could reveal therapeutic targets for speeding wound healing. Polyphosphate is present in human wounds along with monocytes, neutrophils, and peripheral blood mononuclear cells (Smith, Mutch et al. 2006).

We propose to test the effect of polyphosphate on the chemotaxis of monocytes, neutrophils, and the model organism *Dictyostelium discoideum*. To quantify chemotaxis, we will film the movement of cells in a gradient of polyphosphate, and measure the magnitude and speed of cell movement.

Polyphosphate does not significantly affect either *Dictyostelium*, neutrophil, or monocyte forward migration index. However, when polyphosphate was added to *Dictyostelium* and

neutrophils a statistically significant effect on their speed was measured. This could warrant further research into questioning why the speed was affected by polyphosphate.

DEDICATION

I would like to dedicate this thesis to family and friends, especially my father Eugenio Roberto Chinaa and my mother Elizabeth Chinaa. I would also like to thank my friends, for supporting me in writing this thesis.

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I would like to thank Dr. Richard Gomer and all the members of his lab. I want to specifically thank Dr. Michael White for mentoring me when I first joined Dr. Gomer's lab.

I would also like to thank the nurses at the Beutel health center who performed the blood draws for the experiments in this thesis.

Lastly, I would like to thank my family for all the support and encouragement they have given me while writing this thesis.

NOMENCLATURE

PBMC	Peripheral blood mononuclear cells
AprA	Autocrine proliferation receptor
SIH	Defined minimal media
FMI	Forward migration index
Poly-p	Polyphosphate
PBM	Phosphate buffer media

CHAPTER I

INTRODUCTION

The role of polyphosphate

It is not well understood why immune cells are brought together to the site of a wound. Wound treatment in the United States costs \$25 billion annually (Sen, Gordillo et al. 2009) and one in a hundred people are hospitalized each year for a wound that requires professional medical attention (Drew, Posnett et al. 2007, Srinivasaiah, Dugdall et al. 2007, Hurd and Posnett 2009, Vowden and Vowden 2009, Vowden and Vowden 2009). Wound healing is a dynamic process that involves several different cell types, from local epithelial cells and fibroblasts to circulating immune system cells, such as neutrophils and monocytes. Polyphosphate is present in human wounds along with monocytes and neutrophils (Smith, Mutch et al. 2006).

Long chains of inorganic phosphate molecules, known as polyphosphate, are found in healing wounds, but our lab has also identified polyphosphate as a molecule of interest in *Dictyostelium* biology. *Dictyostelium discoideum* is a unicellular model organism that is useful for modeling the developmental processes of chemotaxis and proliferation. Polyphosphate is secreted by *Dictyostelium discoideum* as a developmental factor and is secreted by platelets when platelets are attracted to the site of a human wound (Smith, Mutch et al. 2006).

The effect of polyphosphate in a wound

Polyphosphate is stored in the acidocalcisome organelle found in platelets. When platelets get activated polyphosphate is secreted, enhancing clot formation and delay clot lysis by promoting a

natural antifibrinolytic agent (Smith, Mutch et al. 2006). The function of polyphosphate has not been thoroughly studied in higher eukaryotes. The effects that polyphosphate has on neutrophils and peripheral blood mononuclear cells has not been completely elucidated.

CHAPTER II

METHODS

Peripheral blood mononuclear cell and neutrophil isolation

With approval from the Institutional Review Board of Texas A&M University, blood was collected from healthy volunteers at the Beutel Student Health Center. All blood samples obtained had given written consent and were deidentified before analysis. Neutrophils were isolated from the blood samples by using Lympholyte-poly (Cedarlane Laboratories, Burlington, Canada) following the manufacturer's instructions except the samples were centrifuged for 40 min instead of 30-35 min at 500g, and re-suspended in 2% bovine serum albumin (BSA; AMRESCO, Ohio, United States of America) in RPMI-1640 (Lonza) (Herlihy et al. 2013). Peripheral blood mononuclear cells were isolated from the blood samples by using Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) following the manufacturer's instructions and re-suspended in 2% BSA (AMRESCO) in RPMI-1640 (Lonza).

***Dictyostelium discoideum* cell culture**

Dictyostelium discoideum Ax2 wild type strain was used. The strain was acquired from frozen stocks that were ordered from dictybase.org. The Ax2 wild type were grown on lawns of bacteria, then moved into axenic shaking cultures using HL5 growth media (Formedium) supplemented with streptomycin or SIH media (Formedium) (Brock et al. 1969). Fresh cultures were started every 3-4 weeks.

Insall chamber assay

Polyphosphate purchased from Spectrum was used and are approximately 70 phosphate residues long. The polyphosphate was filter sterilized into the appropriate media. An Insall chamber was used to measure Neutrophil and PBMC displacement caused by polyphosphate (Muinonen-Martin et al. 2010). Chemotaxis assays were performed as described previously (Herlihy et al. 2013) except instead of dipeptidyl peptidase IV, polyphosphate was used and analysis of the data was done differently. After filming cells in the insall chamber for 1 hour at 37C in a humidified 5% CO₂ incubator, displacement of a minimal of 10 randomly chosen cells per experiment were analyzed. Tracking analysis was performed by taking the hour long video and using Virtual Dub to break the video into approximately 240 images with each image being an interval of 15 seconds. Then the 240 jpeg images were put into the software ImageJ where the images were stacked upon each other. Once the images were stacked, at least 10 random cells were manually tracked for each experiment's set of images. ImageJ then gives data on how far each cell was displaced in pixel value. Pixel data was inputted into the program Ibidi Chemotaxis and Migration Tool which converted the pixel displacement into micron displacement. By measuring in microns how wide the image is and how many pixels are in each image, we were able to determine the pixels per micron conversion which we put into Ibidi. This then gives the distance in microns that each cell travels. After calculating the displacement of each cell in microns through this pipeline, the displacement data was put into the program GraphPad Prism where analysis of the data took place and statistical values were calculated. To measure the effect polyphosphate has on *Dictyostelium discoideum* an Insall Chamber was utilized as described by (Phillips et al. 2012) except instead of AprA, polyphosphate was utilized. The analysis of the data was done differently. Briefly, approximately 1.5 mL of 50,000 to 75,000 cells/mL was used

per experiment. Cells were taken from the shaking culture and counted on the hemocytometer. The cells were tracked and filmed similar to the procedure for neutrophils and PBMC's except instead of taking a 1 hour video of the cells, jpeg images were taken of the cells, with a Nikon Microphot FX microscope and an OMAX 10 megapixel camera with the program ToupView, every 15 seconds for 1 hour.

CHAPTER III

RESULTS

The effect polyphosphate has on the FMI of *Dictyostelium discoideum*

The data from figure 1 suggests, there is a slight chemo-attraction of *Dictyostelium* cells with 600 μ M and 150 μ M of polyphosphate. However, when applying an unpaired t-test, both concentrations of polyphosphate did not display a statistical significance in their FMI's (figure 1). The data from figure 2 shows that there is not a statistical significance in the SIH cultured *Dictyostelium* cells FMI values. The t-test indicated that polyphosphate at 150 μ M is not having a statistically significant effect on the FMI of starved *Dictyostelium* cells (figure 3).

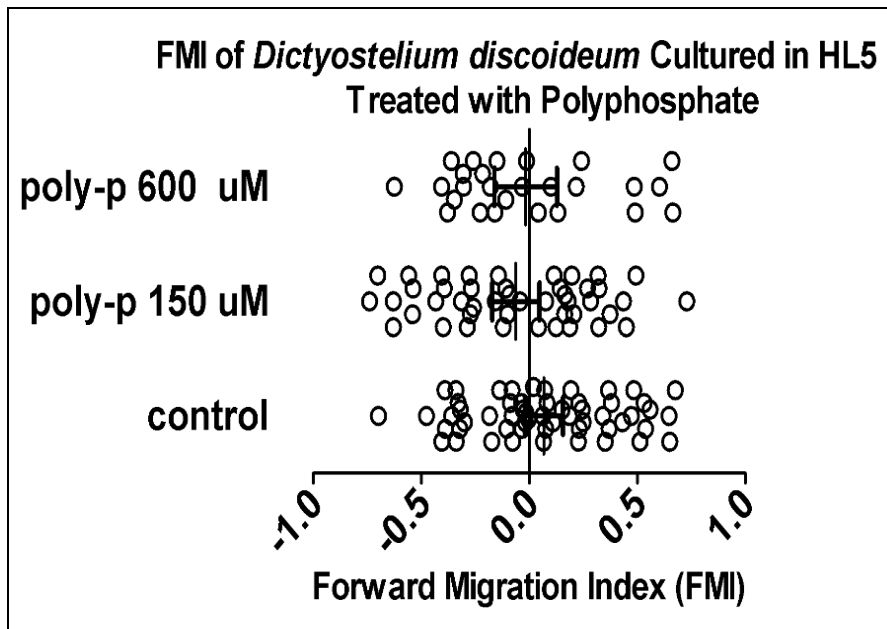


Figure 1: FMI of *Dictyostelium discoideum* cultured in HL5 media treated with polyphosphate. An insall chamber assay was used to acquire FMI data. The data shows the FMI for *Dictyostelium discoideum* cultured in HL5 media, when polyphosphate is added at 600 μ M, 150 μ M, and without polyphosphate. The results from the FMI data are based on the analysis of $n \geq 3$ [mean \pm interquartile mean, $p = 0.2978$, $p = 0.0624$ t-test].

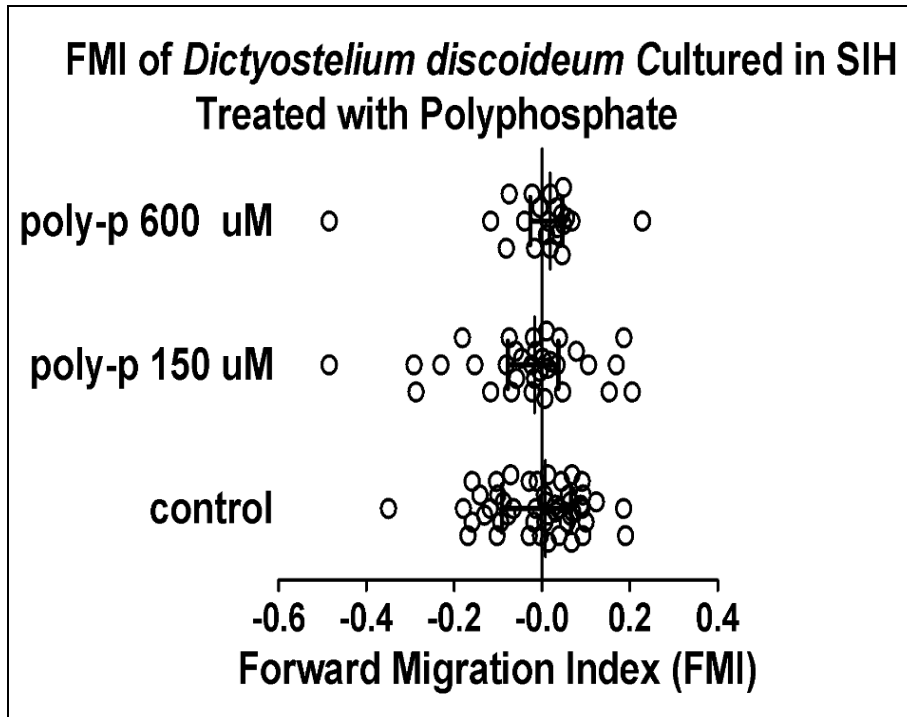


Figure 2: FMI of *Dictyostelium discoideum* cultured in SIH media treated with polyphosphate. An insall chamber assay was used to acquire FMI data. The data shows the FMI for *Dictyostelium discoideum* cultured in SIH media, when polyphosphate is added at 600 μ M, 150 μ M, and without polyphosphate. The results from the FMI data are based on the analysis of $n \geq 3$ [mean \pm interquartile mean, $p = 0.9118$, $p = 0.3465$ t-test].

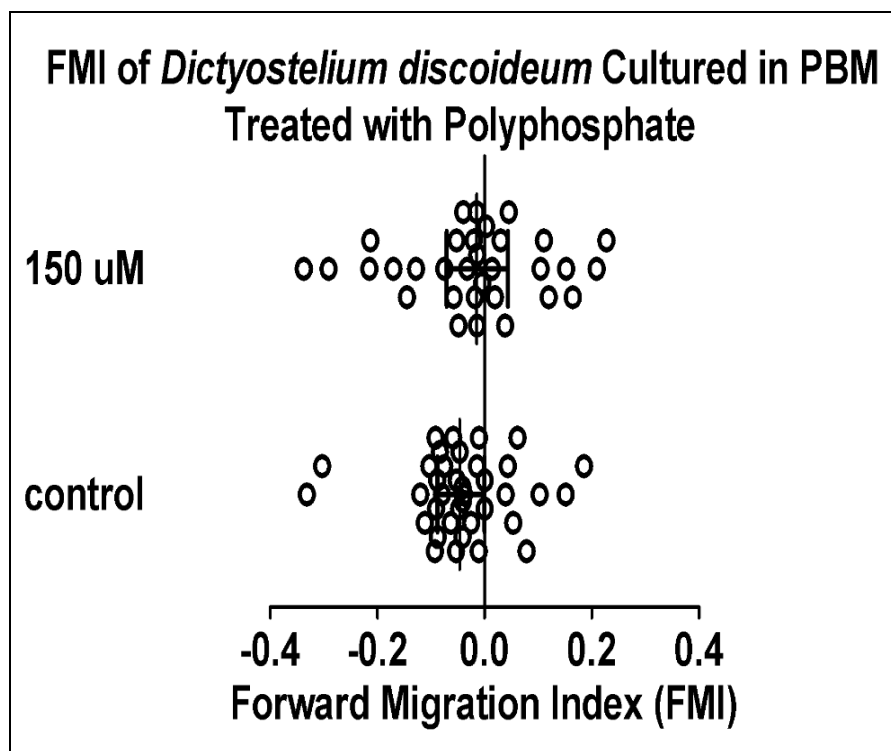


Figure 3: FMI of *Dictyostelium discoideum* starved in PBM media treated with polyphosphate. An insall chamber assay was used to acquire FMI data. The graphs show FMI data for *Dictyostelium discoideum*, starved for approximately 6 hours in PBM media, when polyphosphate is added at 150 μ M and without polyphosphate. The results from the FMI data are based on the analysis of $n \geq 3$ [mean \pm interquartile mean, $p = 0.2308$ t-test].

The effect polyphosphate has on the speed of *Dictyostelium discoideum*

The t-test revealed that there was not a statistically significant effect, when polyphosphate was added to *Dictyostelium* cells cultured in HL5 media (figure 4). Figure 5 shows that when polyphosphate is added at 150 μ M the speed of *Dictyostelium* cells increases and after performing an unpaired t-test the difference in speed change compared to the control is statistically significant. Figure 5 also shows that when polyphosphate is added to *Dictyostelium* cells at 600 μ M their speed decreases significantly and after performing an unpaired t-test the difference in speed is statistically significant. The data in figure 6 indicates that the speed of *Dictyostelium*

cells is affected by 150 μ M of polyphosphate. This was proven to be statistically significant by the t-test.

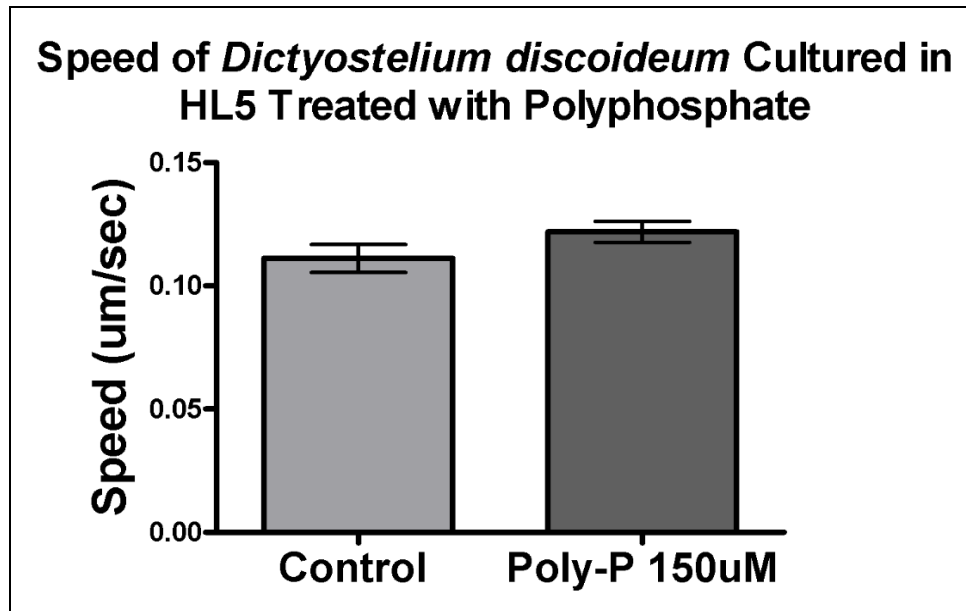


Figure 4: Speed of *Dictyostelium discoideum* cultured in HL5 media treated with polyphosphate. An insall chamber assay was used to acquire speed data. The effect that polyphosphate has on the speed of *Dictyostelium discoideum* cultured in HL5 media with polyphosphate added at 150 μ M and without polyphosphate. The results from the speed data are based on the analysis of $n \geq 3$ [mean \pm SEM, $p = 0.1595$ t-test].

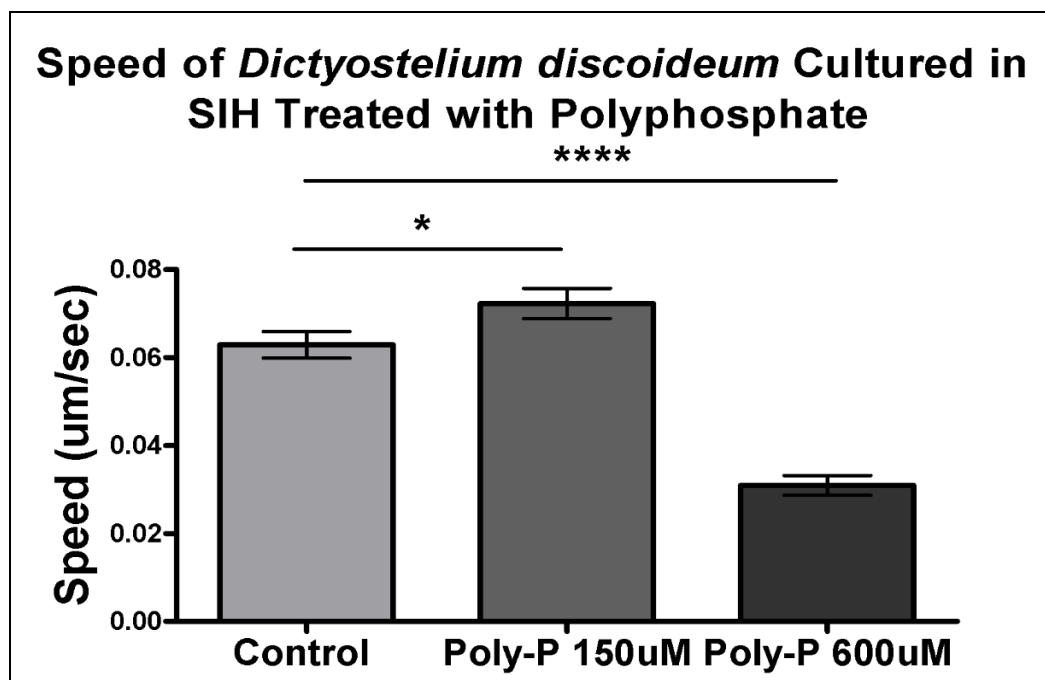


Figure 5: Speed of *Dictyostelium discoideum* cultured in SIH media treated with polyphosphate. An insall chamber assay was used to acquire speed data. The effect that polyphosphate has on the speed of *Dictyostelium discoideum* cultured in SIH media with polyphosphate added at 150 μ M and 600 μ M and without polyphosphate. The results from the speed data are based on the analysis of $n \geq 3$ [mean \pm SEM, $p < 0.05$, $p = 0.0001$ unpaired t-test and $p < 0.0001$ one-way ANOVA].

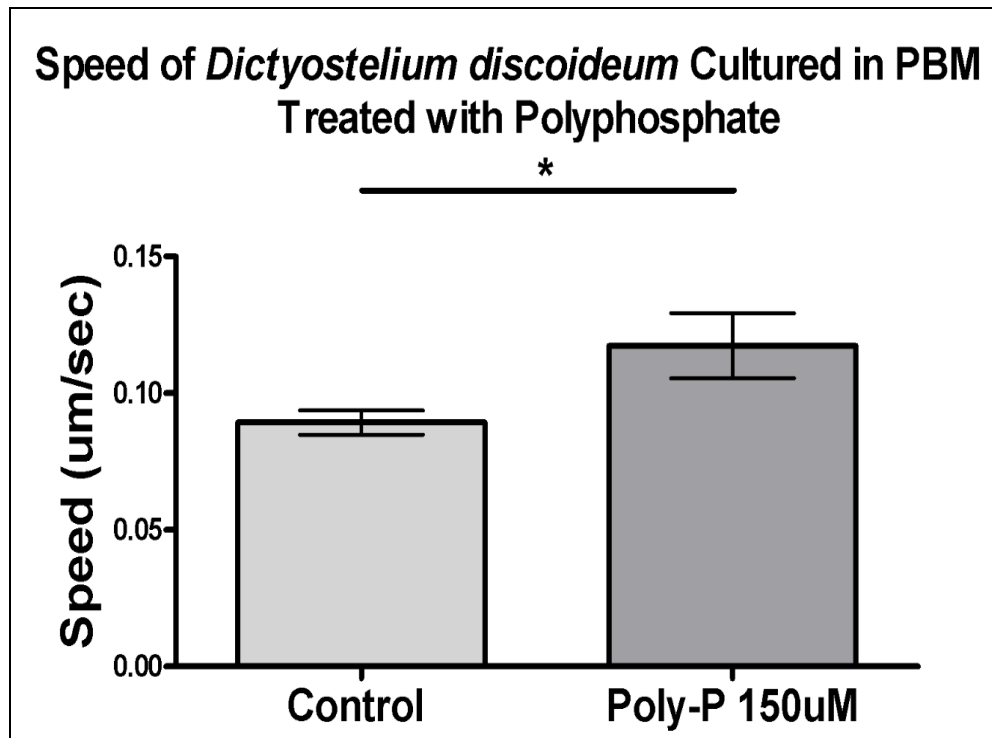


Figure 6: Speed of *Dictyostelium discoideum* starved in PBM media treated with polyphosphate. An insall chamber assay was used to acquire speed data. The charts show speed data for *Dictyostelium discoideum*, starved for approximately 6 hours in PBM media, when polyphosphate is added at 150 μ M and without polyphosphate. The results from the speed data are based on the analysis of $n \geq 3$ [mean \pm SEM, $p < 0.05$ t-test].

The effect polyphosphate has on the FMI of Neutrophils and PBMC's

When neutrophils are treated with polyphosphate at 200 μ M, the FMI is not affected to a statistically significant extent (figure 7). Figure 8 shows that PBMC's treated with 200 μ M of polyphosphate do not show FMI bias to a statistically significant extent.

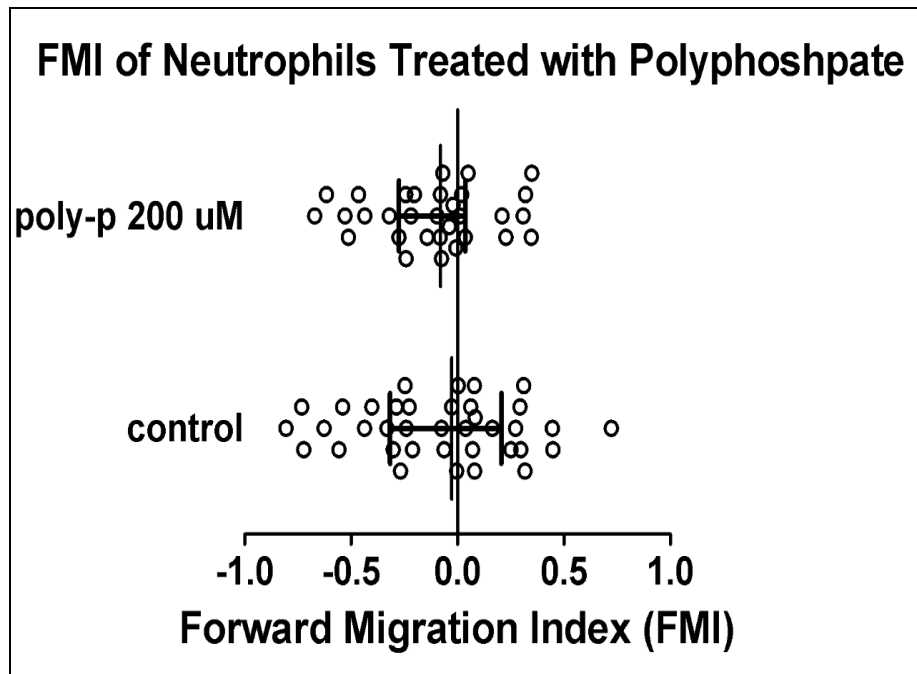


Figure 7: FMI of neutrophils treated with polyphosphate. An insall chamber assay was used to acquire FMI data. The chart shows the FMI of neutrophils when treated with polyphosphate at 200 μ M and without polyphosphate. The results from the FMI data are based on the analysis of $n \geq 3$ [mean \pm interquartile mean, $p = 0.747$ t-test].

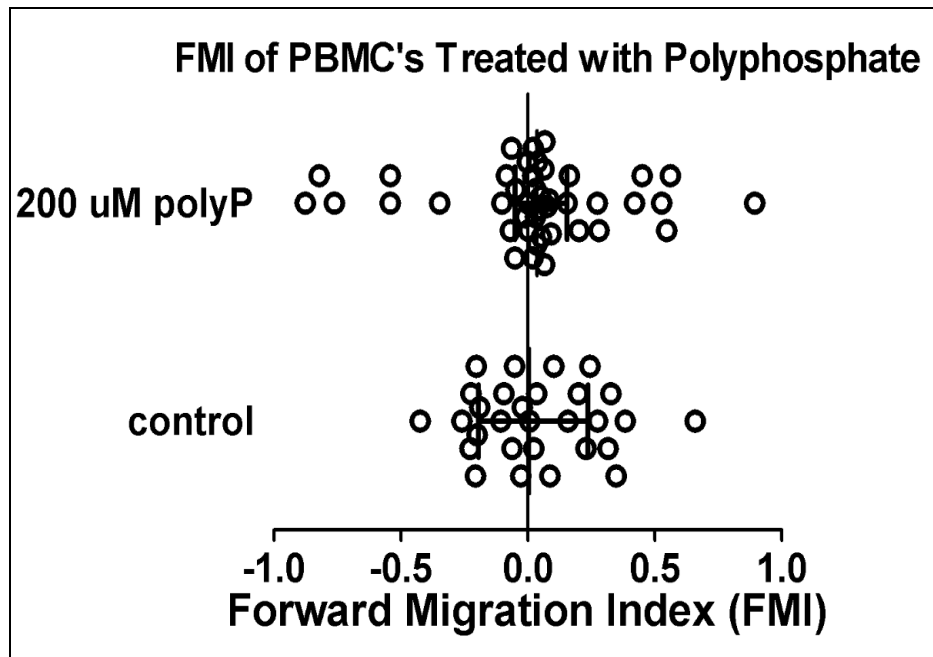


Figure 8: FMI of PBMC's treated with polyphosphate. An insall chamber assay was used to acquire FMI data. The chart shows the FMI of PBMC's when treated with polyphosphate at 200 μ M and without polyphosphate. The results from the FMI data are based on the analysis of $n \geq 3$ [mean \pm interquartile mean, $p = 0.3762$ t-test].

The effect polyphosphate has on the speed of Neutrophils and PBMC's

The speed of neutrophils decreased to a statistically significant degree, when treated with polyphosphate at 200 μ M (figure 9). PBMC's treated with polyphosphate at 200 μ M do not display an affected speed (figure 10).

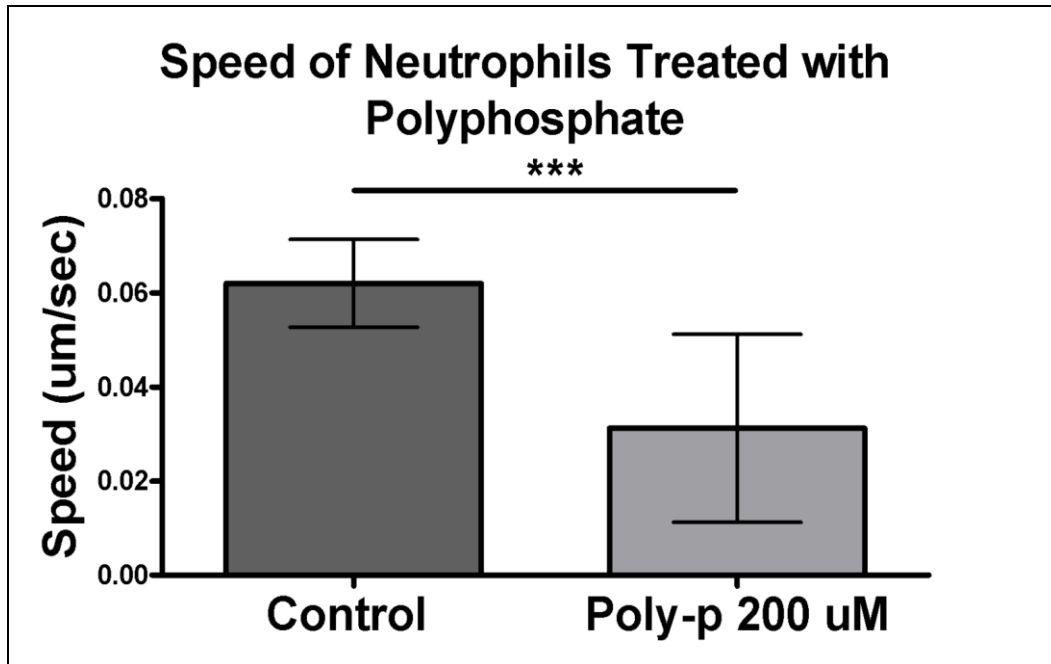


Figure 9: Speed of neutrophils treated with polyphosphate. An insall chamber assay was used to acquire speed data. The chart shows the speed of neutrophils when treated with polyphosphate at 200 μ M and without polyphosphate. The results from the speed data are based on the analysis of $n \geq 3$ [mean \pm SEM, $p = 0.0009$ t-test].

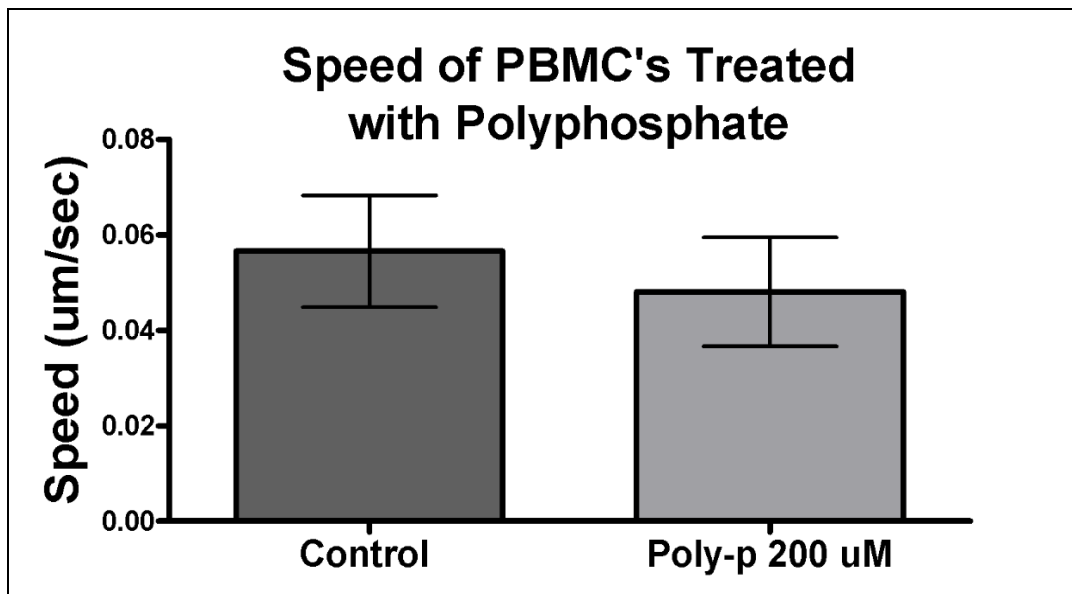


Figure 10: Speed of PBMC's treated with polyphosphate. An insall chamber assay was used to acquire speed data. The chart shows the speed of PBMC's when treated with polyphosphate at 200 μ M and without polyphosphate. The results from the speed data are based on the analysis of $n \geq 3$ [mean \pm SEM, $p = 0.6084$ t-test].

CHAPTER IV

CONCLUSION

Effects of polyphosphate on neutrophils and PBMCs

Polyphosphate in humans has previously been characterized as an agent that is secreted by platelets upon activation and enhances clot formation and delays clot lysis by promoting a natural antifibrinolytic agent (Smith, Mutch et al. 2006). In these experiments we further analyzed the role of polyphosphate in a wound to test if polyphosphate has an effect in biasing chemotaxis of neutrophils and PBMCs. We found that polyphosphate does not have a role in biasing chemotaxis of neutrophils and PBMCs however, it does slow the speed of neutrophils.

In an Insall chamber, we observed that the FMI of neutrophils and PBMCs was not affected by a polyphosphate gradient. Polyphosphate is secreted by platelets once they are activated in a wound and the findings from these experiments show that polyphosphate at 200 μ M is not responsible for the recruitment or repulsion of neutrophils and PBMCs. The speed of the neutrophils was affected by polyphosphate. The reason why the speed of neutrophils was slower with the addition of polyphosphate warrants further research into what interactions take place between polyphosphate and neutrophils.

Effects of polyphosphate on *Dictyostelium discoideum*

Polyphosphate has a role in *Dictyostelium discoideum* as a secreted developmental factor. With the results of these experiments, we know that polyphosphate does not play a role in biasing the direction of *Dictyostelium discoideum* cell movement. Our results show that polyphosphate

affects the speed of *Dictyostelium discoideum* cultured in SIH and PBM media. When 150 μM polyphosphate was added to *Dictyostelium* cells, they responded by increasing their speed compared to the controls. In SIH, a ~16% increase in speed was observed when polyphosphate was added to *Dictyostelium* cells, and a ~33% increase in speed when cultured in PBM. Further research into why polyphosphate increases the speed of *Dictyostelium discoideum* could be done to identify what is interacting with polyphosphate to cause an increase in the speed of the cells.

When 600 μM polyphosphate was added to *Dictyostelium discoideum* cells cultured in SIH, they responded by decreasing their speed ~50% compared to the controls. The decrease in speed could be due to the high concentration of polyphosphate added. In our lab, polyphosphate has been shown to be toxic at a concentration greater than 200 μM . The decrease in speed from the *Dictyostelium discoideum* cells could be a direct response to the cells dying from the toxic concentration of polyphosphate.

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